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Studies on the base pairing properties of deoxyinosine by solid phase hybridisation to oligonucleotides

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ABSTRACT

Extensive analyses of the base-pairing properties of deoxyinosine to A, C, G and T were carried out by measuring the hybridisation of oligonucleotides with deoxyinosine in various positions to complementary sets of oligonucleotides made as an array on the surface of a glass microscope slide. With deoxyinosine in internal positions, results are consistent with previous studies, showing a preferential order for pairing of I-C > I-A > I-G ~ I-T. With two adjacent deoxyinosines in the centre of the oligonucleotide, the order in duplex yield is CC > CA > AA > AC > GC > GA > CG > TA > TC > CT = AG > AT > GT > TT. With deoxyinosine at the ends of the oligonucleotide, we find that at the 3′ end there is the same order in duplex yield as for the deoxyinosine in internal positions, though with lower discrimination between the bases. When hybridisation is carried out in TMACl there is little base pairing discrimination with deoxyinosine, or indeed any of the four natural bases at the 5′ end. Changing the cation to Na^+ increased discrimination slightly.

INTRODUCTION

A universal base which pairs equally well with the four naturally occurring bases would find many applications in molecular biology (1). For example, the complexity of mixed oligonucleotide probes and primers needed to deal with degenerate codons would be greatly reduced.

The nucleoside most widely used for this purpose is inosine, which occurs in the wobble position of some tRNA anticodons, where it pairs with adenosine cytidine and uridine. It has a similar structure to guanosine, but lacks the 2-amino group, and pairs with the natural bases as shown in Figure 1 (2–9). Deoxyinosine has been investigated as a universal base for use in oligonucleotide probes and primers (10–12). Martin *et al.* using an optical melting technique concluded that it does not pair equally with the four naturally occurring bases, the thermodynamic stabilities being in the order I-C > I-A > I-G ~ I-T. Furthermore, neighbouring groups were found to have large effects on stability, though crystal structure evidence has shown that duplexes containing deoxyinosine do not show large perturbations at a local level. Several synthetic bases have been designed for degenerate pairing. For instance the base 6H,8H-3,4-dihydropyrimido[4,5-c][1,2]oxazin-7-one pairs with both A and G, and the base 2-amino-6-methoxyaminopurine pairs with C and T (13, 14). These bases have been used successfully for partial substitution of natural bases in probes and primers, allowing some reduction in complexity.

Degenerate bases could also be useful in nucleic acid sequence determination by hybridisation to oligonucleotides (15, 16). In this method a set of oligonucleotides of defined sequence is hybridised to the nucleic acid of unknown sequence. The sequence is reconstructed from the sequences of the oligonucleotide components identified by hybridisation. Theory shows that longer sequences can be reconstructed from a set of a given size, if the oligonucleotides are extended by degenerate bases in internal positions (17, 19). For example, by adding fully degenerate bases in two positions of an array of all hexanucleotides, the maximum length that can be sequenced increases from about 50 to about 200 bases. Theory suggests then that the size of the oligonucleotide set can be decreased by one sixteenth to achieve the same performance. In the work described in this paper, we used array-based methods to study the hybridisation behaviour of deoxyinosine for possible use in sequencing by hybridisation to oligonucleotides; and although we used tethered oligonucleotides the results should be relevant to any application of oligonucleotide hybridisation which might benefit from the use of a universal base.

MATERIALS AND METHODS

3-Glycidoxypropyltrimethoxysilane, hexaethylene glycol and HPLC grade acetonitrile were purchased from Aldrich and were used without further purification. All DNA synthesis materials were purchased from Applied Biosystems.

Synthesis of the linker on the glass surface was carried out as described (20). A solution of 5% 3-glycidoxypropyltrimethoxysilane in water was adjusted to a pH between 5.5 and 5.8 by addition of 1 mM potassium hydroxide. Glass plates were immersed in this solution, heated to 90°C and held at this temperature for 30 min after which the plates were washed with water and acetone. (We have subsequently found that more complete derivatisation is achieved using a solution of glycidoxypropyltrimethoxysilane in xylene containing a trace of diisopropylethylamine, according to the previously published method.) In a second step the plates were heated in neat hexaethylene glycol containing a catalytic amount of sulphuric acid at 80°C overnight. After washing with methanol and ether the plates were vacuum dried and stored at −20°C.

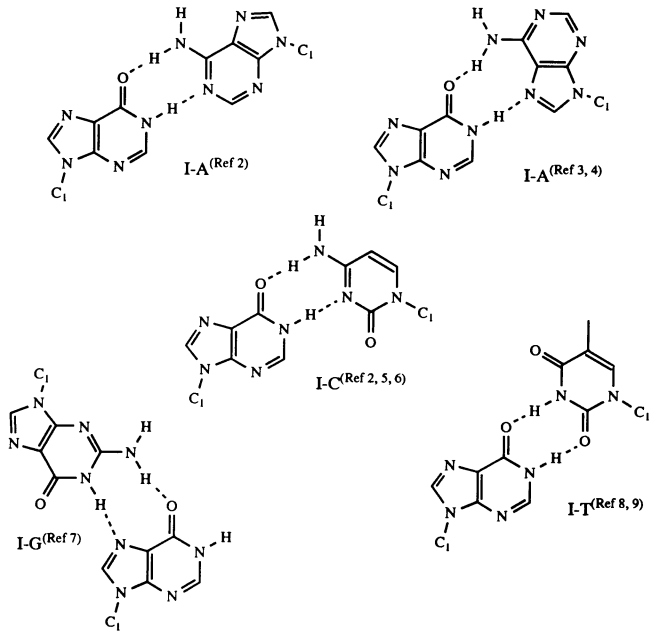

Figure 1. Proposed configurations for base pairs containing inosine.

One of two methods was used to synthesise the oligonucleotides on the glass surface. A flat helix was produced by weaving polypropylene tubing (0.5 mm o.d.) between the teeth of two combs held ca. 10 cm apart in a frame; the pitch of the helix was 3 mm or 6 mm so that when the tubing was clamped between two microscope slides it created channels 3 mm×0.5 mm or 6 mm×0.5 mm. These channels were used to introduce reagents to the surface of the microscope slides, after thorough gassing with dry argon. To ensure precise registration of the channels, one side of the slides was placed against a stop fixed at one side of the template. As the ends of the polypropylene tubing extended past the ends of the slides it was not necessary to locate the front and back edges of the slide. Hydrogen phosphonate chemistry was used for the synthesis of the oligonucleotides (21). Precursor and activator solutions were held in two 10 ml syringes that were driven simultaneously by an infusion pump. The syringes were connected by Teflon tubing to sawn off syringe needles, and reagents injected into the channels simultaneously; mixing occurred as the reagents entered the channels. After coupling for a minimum of 1 min, the reagents were removed and the channels washed with acetonitrile. No capping step was used. The slides were separated and immersed in dichloroacetic acid (2.5% in dichloromethane) for 100 secs, washed in acetonitrile and dried in a stream of argon. The slides were then ready for another round of coupling.

For synthesis over the entire surface, two plates were clamped face to face with their edges sealed by a gasket cut from a sheet of silicon rubber (0.5 mm thick), with one end open to permit injection of reagents. Solutions were introduced as described above, rocking the assembly to ensure good mixing. Other steps were as described above.

As the amount of oligonucleotide made on a plain glass surface is small, trityl yields are too low to estimate coupling efficiency. For this reason, we made six copies of each oligonucleotide set to give multiple analyses.

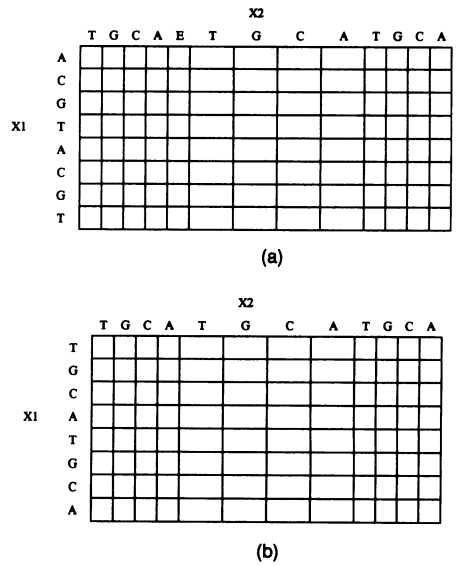

Figure 2. a. Protocol for applying base precursors during the synthesis of array 1, to produce six copies of the sixteen oligonucleotides of formula 3′ $GGGX_1X_2AAA$ on the surface of a microscope slide. Precursors for the first three and last three bases were applied over the entire surface. Bases X_1 and X_2 were applied through channels, and in the order indicated by the rows and columns. In the middle two sets the base X_2 was added in a band twice the width of the outer two sets. The column marked E indicates that no X_2 base was added, so that the oligonucleotides in these positions are heptamers. The squares/rectangles in the drawing outline an individual cell of the array. **b.** Position of oligonucleotides in array 2. The bases specifying individual cells in the array were applied at the beginning and the end of the synthesis to produce six copies of the sixteen oligonucleotides of formula $3'X_1GGGAAAX_2$. The squares/rectangles in the drawing outline an individual cell of the array. Again in the middle two sets the base X_2 was added in a band twice the width of the outer two sets.

Oxidation of the hydrogen phosphonate linkage was performed in a single step at the end of the synthesis, using reagents and conditions supplied by the manufacturer, in the set up described above. The slides were washed in acetonitrile and dried under an argon stream. Deprotection was carried out in a sealed glass bottle (Schott) using 30% ammonia at 55°C for 5−10 h.

Oligonucleotides containing deoxyinosine for use as probes were synthesised on an Applied Biosystems model 381A, using standard phosphoramidite chemistry and purified using ABI purification cartridges (22). After end-labelling with polynucleotide kinase and γ^{32}P-ATP, and removal of excess γ^{32}P-ATP, an aliquot was used for hybridisation to the slides. The solvent was either 3.5 M tetramethylammonium chloride (TMACl) containing 50 mM Tris−HCl, pH 8.0, 2 mM EDTA and SDS at less than 0.04 mg/ml or 1 M sodium chloride (STE) containing 10 mM Tris−HCl, pH 8.0, 1 mM EDTA and SDS at less than 1 mg/ml. After hybridisation, the slides were washed for 1 min in the same solvent at the hybridisation temperature, sealed in Clingfilm and exposed to a storage phosphor screen (Fuji, STIII) at the same temperature as used for hybridisation. The screens were scanned in a PhosphorImager 400A (Molecular Dynamics). Relative duplex yields were measured, using ImageQuant software, by one of two methods: a grid was superimposed on the array and pixel values in each cell integrated with automatic background subtraction. Alternatively, a rectangle was drawn to encompass the cells in one column or row; pixel lines were integrated across the rectangle to generate a 1-D

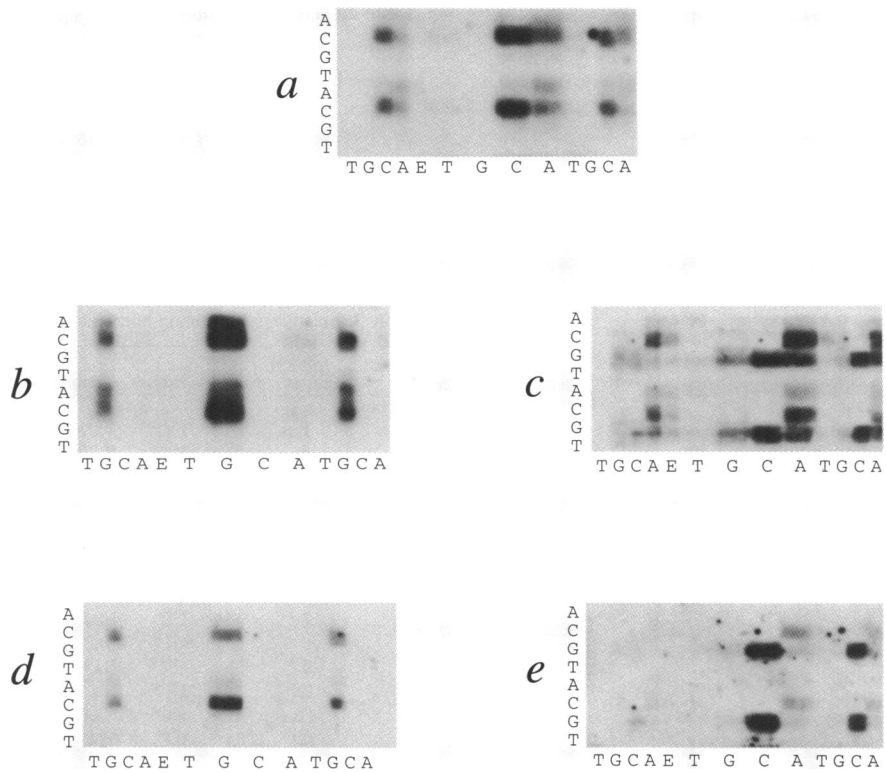

Figure 3. Results of hybridising oligonucleotides to array 1. All hybridisations were carried out for 5 hours at 4°C in either in either 3.5 M TMA or 1 M STE (**a**) I^5I^6, TMA (**b**) I^5C^6, TMA, (**c**) C^5I^6, TMA (**d**) I^5C^6, STE (**e**) C^5I^6, STE.

profile, and areas under peaks integrated in a second step. Several values for each sequence were obtained from different cells, and mean and standard deviations calculated. The data showed greatest consistency in the large cells in the middle sections of the array. This may be due to the reduction in the relative amounts of shine from ^{32}P in adjacent cells of the grid.

RESULTS

An advantage of using arrays of oligonucleotides for comparing reassociation behaviour is that much can be learned from visual inspection of the results. The eye is very good at comparing intensities and especially good at discerning small differences. Some of our experiments show that changing the base at one end of the oligonucleotide on the plate has no effect on duplex formation. This can be seen from the uniform intensity of the lines running along Array 2, when probed with oligonucleotides with I at the 5′ end (Fig. 4a, b, d, e and g).

However, quantitative comparisons can easily be carried out using the analytical software associated with the phosphoimager, and were used to measure the relative extents of duplex formation and the effects of contributory factors in the two sets of experiments decribed below.

Base pairs at internal duplex positions

Pairing of deoxyinosine to bases in internal positions of the oligonucleotide and effects of bases adjacent to deoxyinosine were analysed using an array of all the octamers of sequence 3′ $GGGN_1N_2AAA$, (N_1, N_2 = A, C, G, or T) (Array 1). Six copies of this set of sixteen octamers were synthesised on a microscope slide (Fig. 2a). Oligonucleotides of formulae ACCCIITTTA, (I^5I^6), ACCCNITTTA, (N^5I^6), and ACCCINTTTA, (I^5N^6), (where N = A, C, G, or T) were used

Table 1. Oligonucleotide probes used in the hybridisation studies

Probe sequence	Designation
ACCCiiTTTA	I^5I^6
ACCCaiTTTA	A^5I^6
ACCCciTTTA	C^5I^6
ACCCgiTTTA	G^5I^6
ACCCtiTTTA	T^5I^6
ACCCiaTTTA	I^5A^6
ACCCicTTTA	I^5C^6
ACCCigTTTA	I^5G^6
ACCCitTTTA	I^5T^6
AiCCCTTTiA	I^2I^9
AaCCCTTTiA	A^2I^9
AcCCCTTTiA	C^2I^9
AgCCCTTTiA	G^2I^9
AtCCCTTTiA	T^2I^9
AiCCCTTTaA	I^2A^9
AiCCCTTTcA	I^2C^9
AiCCCTTTgA	I^2G^9
AiCCCTTTtA	I^2T^9

The top set are designed to hybridise with array 1, the bottom set with array 2.

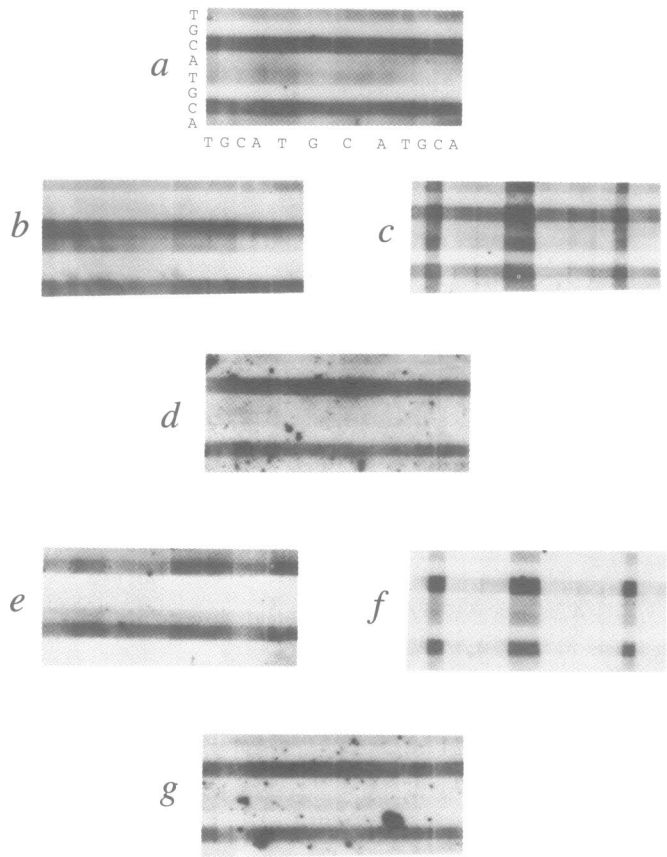

Figure 4. Results of hybridising oligonucleotides to array 2. All hybridisation were carried out for 5 hours at 4°C or 17°C in either 3.5 M TMA or 1 M STE. **(a)** I^2I^9, TMA, 4°C **(b)** T^2I^9, TMA, 4°C **(c)** I^2C^9, TMA, 4°C **(d)** I^2I^9, STE, 4°C **(e)** C^2I^9, STE, 4°C **(f)** I^2C^9, STE, 4°C **(g)** I^2I^9, TMA, 17°C.

as probes (Table 1). (Unless otherwise stated oligonucleotides are written in 5′ to 3′ direction.)

Hybridisation of the oligonucleotide I^5I^6 (3.5 M TMACl at 4°C for 5 h), gave the hybridisation pattern shown in Figure 3a. Relative hybridisation yields, estimated as described in materials and methods, were in the order CC > CA > AA > AC > GC > GA > CG > TA > TC > CT = AG > AT > GT > TT (Table 2). This same trend was seen in the hybridisations of the oligonucleotides ACCCICTTTA, I^5C^6 (Fig. 3b; Table 3, entry1) and ACCCCITTTA, C^5I^6 (Figure 3c).

Interpretation of the hybridisation of C^5I^6 was complicated by the formation of misregistered duplexes. In end to end registration, C^5I^6 should pair through the eight central bases:

```
                A C C C C I T T T A
                – | | | | | | | | –
glass — 3′ – G G G G N A A A –
```

Such pairing would show signals only in the four cells where N_1 = G. However, hybridisation to some cells of the array can be explained by 'staggered' pairing of bases at the 3′ or 5′ ends of the probe oligonucleotide. With slippage towards the 3′ end of the tethered oligonucleotide, there are six Watson–Crick base pairs, an deoxyinosine pairing with cytidine and a terminal A-A mismatch:

Table 2. Relative intensities of of the signal in the cells seen in Figure 3a

	T	G	C	A
A	0.07	0.09	0.30	0.36
C	0.09	0.13	1.00	0.55
G	0.04	0.06	0.26	0.19
T	0.03	0.04	0.10	0.11

Integration of cell intensities was achieved by placing a grid over the image and integrating the signal in the cells of the grid using ImageQuant Software.

Table 3. Relative hybridisation intensities in the images seen in Figures 3 and 4

						Relative Duplex Yield			
	Entry	Figure	Probe	Cation	Temp°C	A	C	G	T
Array1									
	1	3b	I^5C^6	TMA	4	0.53	1.00	0.06	0.04
	2	3d	I^5C^6	Na	4	0.16	1.00	0.03	0.02
	3	-	I^5C^6	TMA	17	0.43	1.00	0.09	0.07
Array2									
	4	5b	T^2I^9	TMA	4	1.00	1.00	0.75	0.75
	5	5c	I^2C^9	TMA	4	0.44	1.00	0.30	0.64
	6	5c	I^2C^9	TMA	4	0.52	0.52	1.00	0.56
	7	5d	I^2I^9	Na	4	0.80	1.00	0.90	0.60
	8	5d	I^2I^9	Na	4	0.33	1.00	0.17	0.22
	9	5e	C^2I^9	Na	4	0.90	1.00	0.48	0.52
	10	5f	I^2C^9	Na	4	0.33	1.00	0.11	0.29
	11	5f	I^2C^9	Na	4	0.13	0.13	1.00	0.19
	12	5g	I^2I^9	TMA	17	0.14	1.00	0.13	0.21
	13	-	I^2C^9	TMA	17	0.35	1.00	0.20	0.45
	14	-	I^2C^9	TMA	25	0.24	1.00	0.19	0.27
	15	-	I^2C^9	TMA	17	0.13	0.13	1.00	0.22
	16	-	I^2C^9	TMA	25	0.28	0.10	1.00	0.28

The columns on the right indicates the base which specifies a cell in the array. The highlighted base in the probe is that which pairs to this base. Yields of duplex are expressed relative to the highest taken as 1. The values in the T column are relatively high because of slippage — see text.

Table 4. Relative intensities of the signal in the cells seen in Figure 4a

	T	G	C	A
T	0.46	0.40	0.37	0.42
G	0.30	0.27	0.25	0.26
C	0.99	0.97	0.98	1.00
A	0.49	0.44	0.44	0.48

```
                A C C C C I T T T A
                – – | | | | | | | –
glass — 3′ – G G G N A A A A
```

Similarly, but to a lesser extent, duplex formation was seen from slippage towards the 5′ end of the tethered oligonucleotides which produces six Watson–Crick base pairs, an I pairing with A and a terminal A-G mismatch:

```
                A C C C C I T T T A
                – | | | | | | | – –
glass — 3′ – G G G G G A A A – –
```

These misregistered pairings complicate the analysis, but taken with other data extend the results to provide information on other base pairing interactions.

In 1 M STE at 4°C I^5I^6 gave no detectable signal and hybridisation of I^5C^6 (Figure 3d) and C^5I^6 (Figure 3e) showed that these conditions are more stringent than hybridisation in TMA. In the presence of sodium ions, the rank was the same,

i.e. I-C > I-A > I-G ~ I-T, but the discrimination was greater (Table 3, entry 2 vs 1). The misregistered pairing seen with C^5I^6 was also much reduced, with only slippage towards the 3′ end of the oligonucleotide making a significant contribution. Hybridisation of I^5I^6 at 17°C in 3.5 M TMA gave no detectable signal. I^5C^6 did hybridise under these conditions (Table 2, entry 3). No oligonucleotide showed significant hybridisation at 17°C in 1 M STE. These results all indicate that all base pairs are less stable in 1 M NaCl than in 3.5 M TMA, and that this lowered stability results in greater discrimination between paired and mispaired bases. Taken together, these results also show that base pairs between I and any of the four bases are weaker than both Watson–Crick base pairs.

Base pairing of deoxyinosine in terminal positions

A second array (Array 2) was designed to examine the pairing of deoxyinosine in terminal positions. This array comprised six copies of the set of sixteen octamers of sequence 3′-N_1GGGAAAN_2, (N_1, N_2 = A, C, G, or T) (Fig. 2b). Oligonucleotides of formulae AICCCTTTIA, (I^2I^9), AICCCTTTNA, (I_2N_9) and ANCCCTTTIA, (N_2I_9) (where N = A, C, G or T) were used as probes (Table 1).

Hybridisation of the oligonucleotide AICCCTTTIA, (I_2I_9), in 3.5 M TMA (Fig. 4a and Table 4) showed that the position of the deoxyinosines significantly affected the extent of pairing. Deoxyinosine at the 3′ end of the oligonucleotide shows the same signal strength when paired against any of the four bases, but at the 5′ end of the oligonucleotide, inosine preferentially pairs in the order seen with deoxyinosine in internal positions, but with reduced discrimination. Interpretation is complicated by the a contribution in the line corresponding to pairing with thymidine due to misaligned pairing of the eight bases at the 5′ end of the oligonucleotide I_2I_9:

```
                A  I  C  C  C  T  T  T  I  A
                |  |  |  |  –  |  |  |  –  –
glass — 3′      T  G  G  G  A  A  A  A  –  –
```

The low relative signal seen in the line corresponding to pairing with guanosine shows that there is little or no misaligned pairing involving the eight bases at the 3′ end of the tethered oligonucleotide:

```
                A  I  C  C  C  T  T  T  I  A
                –  –  |  |  |  |  |  |  |  |
glass — 3′      –  –  G  G  G  G  A  A  A  T
```

Similar patterns of hybridisation were seen with oligonucleotides of formulae ANCCCTTTIA, N_2I_9 and AICCCTTTNA, I_2N_9. For example, the probes ATCCCTTTIA, T_2I_9 (Fig. 4b; Table 3, entry 4) and AICCCTTTCA, I_2C_9 (Fig. 4c; Table 3, entry 5). In this second hybridisation, data for base pairing of cytosine showed that any base at the 3′ end of the tethered oligonucleotide provides little discrimination, by contrast with the 5′ end.

The effect of cation on the base pairing of deoxyinosine at the ends of duplexes was also investigated. In 1 M NaCl, trends were similar to those seen for base pairs in internal duplex positions. For example, hybridisation of I_2I_9 in 1 M STE at 4°C (Fig. 4d; Table 3, entries 7 and 8). This same trend was seen in other hybridisations e.g. ACCCCTTTIA, C_2I_9 (Fig. 4e; Table 3, entry 9) and AICCCTTTCA, I_2C_9 (Fig. 4f; Table 3, entry 10). The specificity of base pairing of the natural bases also increases in sodium as compared with TMA, see for example the pariring of cytosine (Fig. 4f; Table 3, entry 11 vs 2).

Hybridisation in TMA at 17°C was compared with hybridisation at 4°C. Hybridisation of I_2I_9 (Fig. 4g) showed little discrimination in base pairing of the deoxyinosine at the 3′ end of the solution probe at either temperature.

However, deoxyinosine at the 5′ end showed greater discrimination at the higher temperature (Table 3, entry 12). This trend of greater specificity at increased temperature was also seen in the hybridisations of the oligonucleotide I_2C_9, both for I (Table 3, entry 5 vs 13 vs 14) and for C (Table 3, entry 6 vs 15 vs 16).

DISCUSSION

Oligonucleotide arrays enable extensive comparisons of hybridisation reactions in a small number of simple experiments. The approach has a number of advantages. All reactions are done together, eliminating variations in reaction conditions. The flat surface of the glass permits measurements of radioactivity by phosphorimaging, so that subtle differences in hybridisation and problems such as hot-spots in the background are readily seen in the image.

The studies described here extend earlier work with solution hybridisation (10–12), which were carried out close to thermodynamic equilibrium, and measured T_m or T_d. Our measurements were carried far from equilibrium, in a two phase system, using low concentrations of oligonucleotide in the solution phase, at a temperature well below those used in the solution studies, and most measurements were done in TMA^+ rather than Na^+. Measurements were made early in the forward reaction, when few of the tethered oligonucleotides have formed duplex. Nevertheless in agreement with the solution measurements which show that strength of pairing is I-C > I-A > I-G ~ I-T, we find that the duplex yield in the same order when I is paired against one of the natural bases. Ratios vary according to the position of deoxyinosine in the duplex. In internal positions, pairing of I to T or G reduces the hybrid yield 10–30 fold as compared with pairing I to C, and is such as to abolish duplex formation almost completely, even under relatively non-stringent hybridisation conditions. With I at the ends of the duplex, there is a marked difference between one end and the other. At the end which places the deoxyinosine away from the glass, that is at the 3′ end of the solution probe, there is no discrimination between the four bases. This is not a specific property of I, as A, C, G, and T also have little influence at this end. By contrast, there is discrimination when the I or any other base at the 5′ end of the probe oligonucleotide is paired with the base at the proximal end of the tethered oligonucleotide.

Since the advantage of including degenerate bases to extend the usefulness of arrays in reading sequences comes from including them in the middle of the oligonucleotides, we conclude that deoxyinosine is not useful for this purpose. However, deoxyinosine may help in stabilising duplexes when oligonucleotides are used as solution probes, but it should be added to the 3′ end, otherwise it will introduce some base discrimination.

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